due was rendered anhydrous by redissolution in dry pyridine and evaporation *in vacuo*, the process being repeated three times. Pyridine (0.5 ml.) and dicyclohexylcarbodiimide (0.103 g.; 0.5 mmole) were added and the solution was kept sealed at room temperature for 4 days. Water (0.5 ml.) was added and after 12 hr. at room temperature, a small amount of dioxane (1 ml.) and concentrated ammonium hydroxide (3 ml.) were added. When the solution had cleared, additional concd. ammonium hydroxide (9 ml.) was added and the solution was shaken for 2 days. The ammonium hydroxide was carefully removed by evaporation, and 80% aqueous acetic acid (2 ml.) was added to the residual solid. After 4 hr. at room temperature, one-half of the solution was applied on a sheet of Whatman 44 paper and the chromatogram developed in Solvent A. The bands corresponding to uridylyl-(3′→5′)-adenosine and uridine-3′ phosphate were eluted with water and estimated spectrophotometrically. The free nucleotide recovered corresponded to 3.75 μmole (11.3%). The dinucleoside phosphate corresponded to 415 optical density units at 260 mμ. Assuming an εmax. of 23,000 for this compound, the yield was 18.05 μmole (54.5%). This product was shown to be pure uridylyl-(3′→5′)-adenosine by the enzymatic tests specified above. The second half of acetic acid solution was chromatographed after 11 hr. at room temperature. The yield of the dinucleoside phosphate thus isolated was 532 optical density units at 260 mμ (23.2 μmole; 70.2%). This sample was found to contain a maximum of 1.5% ribonuclease-resistant material. Analysis for phosphorus showed ϵ/P at 260 mμ to be 23,000. Ultraviolet absorption characteristics, λmax. 259 mμ at *p*H 7.5; λmax. 259 mμ in alkali; λmax. 257 mμ in acid.

(b) **Using 5′-*O*-trityl-2′-*O*-tetrahydropyranyluridine-3′ phosphate.** An anhydrous pyridine solution (3 ml.) of 5′-*O*-trityl-2′-*O*-tetrahydropyranyluridine-3′ phosphate (0.057 mmole) and N^1,N^6,2′,3′-*O*-tetrabenzoyladenosine (0.146 g.; 0.22 mmole) was prepared by the technique described above under (a). Dicyclohexylcarbodiimide (0.103 g.; 0.5 mmole) was added and the sealed reaction mixture kept at room temperature for 4 days. The volume of the solution then was reduced to one-half and a further amount (0.05 g.) of the carbodiimide was added. After another day, water (1 ml.) was added and the mixture kept for 12 hr. Concentrated ammonia was added until opalescence and when the solution cleared, more conc. ammonia was added until the final volume was 50 ml. After a total of 50 hr. at room temperature, the insoluble precipitate was removed by filtration and the clear filtrate and washings were evaporated to dryness *in vacuo*. The residue was dissolved in 50 ml. of 80% acetic acid and the solution kept at room temperature for 18 hr. The acetic acid was removed *in vacuo* and the residue applied, after adjusting *p*H to 8, to the top of a DEAE-cellulose (carbonate) column (50 cm. × 2 cm. dia.). Elution was carried out using a 0.10 *M* triethylammonium bicarbonate gradient. The dinucleoside phosphate was eluted as a broad peak at 0.045 *M* triethylammonium bicarbonate concentration. Assuming an εmax. (260 mμ) of 23,000, the yield was 57%. Assay with spleen phosphodiesterase and pancreatic ribonuclease showed only a trace of material resistant to these enzymes. Using 7.5 optical density units of the dinucleoside phosphate and applying the total on a paper chromatogram, the optical density of the resistant material was too small to be measured spectrophotometrically.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

Spectroscopic Studies of α-Chymotrypsin Catalyzed Reactions. II. Spectral Changes at 290 mμ

BY JOHN F. WOOTTON[1] AND GEORGE P. HESS

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Absorbancy changes of an enzyme, consisting of amino acids only, have been observed during the formation of an enzyme–substrate complex. Evidence is presented that the ultraviolet difference spectrum of acyl-chymotrypsin *versus* chymotrypsin, with a principal peak at 290 mμ, is intimately related to the acylation of the active site of the enzyme. Both chemical and spectroscopic studies indicate that the origin of the spectral changes is an interaction with a tryptophyl residue in acyl-chymotrypsin. This interaction is not found in chymotrypsin. At the present, one of two different types of interaction with a tryptophyl residue in acyl-chymotrypsin is consistent with the data and considered most likely to account for the observed difference spectrum. These are: (1) an internal cross linkage between an amino acid residue and the indole ring of a tryptophyl residue; or (2) the envelopment of one or possibly more tryptophyl residues in a hydrophobic region of the molecule. This latter possibility requires a reversible change in conformation of the enzyme during the formation of the enzyme–substrate complex.

During the course of investigations of the α-chymotrypsin (CT) catalyzed hydrolysis of *p*-nitrophenyl acetate[2] changes in absorption of the enzyme at 290 mμ,[3] which accompany the formation of the enzyme–substrate complex, were observed. These absorption changes have so far been observed in the catalytic reaction of CT with *p*-nitrophenyl acetate, or with diisopropylphosphorofluoridate (DFP), and in the reaction of trypsin with DFP. The difference spectra of acyl-CT *versus* CT appear to be the same whether the acyl group is acetyl or diisopropylphosphoryl (DIP). The characterization and location of the origin of the difference spectrum could be of considerable significance in terms of the elaboration of the mechanism of action of the enzyme and determination of the structure of the active site of the molecule.

Since DIP-CT is stable over a wide *p*H range the difference spectrum of DIP-CT *versus* CT is characterized in these studies. A preliminary report of a part of these investigations has appeared.[4]

The Relation of the Difference Spectra to the Specific Acylation of the Active Site of CT. A. Difference Spectrum of DIP-CT *versus* CT.—The difference spectrum of DIP-CT *versus* CT at *p*H 6.9 is illustrated in Fig. 1. In this experiment approximately three times the stoichiometric amount of DFP was added directly to the sample cuvette. An equivalent aliquot of dilute 2-propanol was added to the reference solution. The characteristic component of this difference spec-

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(2) (a) G. P. Hess and M. A. Marini, *4th Intern. Congr. Biochem. Vienna*, 1958, p. 42; (b) M. A. Marini and G. P. Hess, *Nature*, **184**, 113 (1959); (c) *J. Am. Chem. Soc.*, **81**, 2594 (1959); **82**, 5160 (1960).

(3) J. F. Wootton and G. P. Hess, *ibid.*, **83**, 4234 (1961).

(4) G. P. Hess and J. F. Wootton, *Fed. Proc.*, **19**, 340 (1960); J. F. Wootton and G. P. Hess, *Nature*, **188**, 726 (1960).

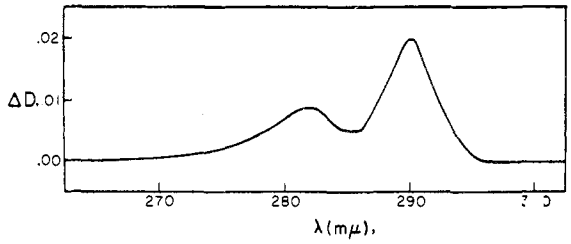

Fig. 1.—Ultraviolet difference spectrum, DIP-CT *versus* CT, *p*H 6.9 (0.033 *M* Tris–HCl, 0.033 *M* $CaCl_2$), 3.6 × 10^{-5} *M* enzyme, 1.1 × 10^{-4} *M* DFP, 25°. Scanned 3 minutes after addition of DFP to sample cell.

trum is a major absorption peak at 290 mμ and a minor peak at 283 mμ.

The reaction of *p*-nitrophenyl acetate[2,5] and DFP[6] with α-chymotrypsin has been thoroughly investigated, and it is well established that these reagents interact with the active site of the enzyme, stoichiometrically, under the experimental conditions employed in the studies reported here. The relationship between the specific reaction of DFP or *p*-nitrophenyl acetate with CT and the difference spectra is established by the following experiments.

(1) The difference spectra are not caused by progressive changes in the CT control solution due to autolysis or denaturation. In the experiment illustrated in Fig. 1 the solutions were scanned three minutes after addition of DFP and at intervals up to twenty-five minutes. No further spectral changes were observed after three minutes. When CT at *p*H 6.9 was compared to *p*H 6.9 buffer, all other conditions being the same as above, spectral changes could *not* be observed during the period examined (25 minutes).

(2) The difference spectra are not due to the reaction of the acylating agents with a contaminant of chymotrypsin preparations. The difference spectrum illustrated in Fig. 1 is obtained in the reaction of DFP with one time crystallized CT, three times crystallized CT, and CT obtained through the activation of twelve times recrystallized chymotrypsinogen.

(3) The difference spectra are not due to the unspecific reaction of DFP with the amino acid side chain of the enzyme. Twelve times recrystallized chymotrypsinogen was treated with a ten fold excess of DFP, employing the conditions of time, *p*H and enzyme concentration used for CT. When the samples were scanned versus a control aliquot of chymotrypsinogen, difference spectra were not observed. When, however, twelve times recrystallized chymotrypsinogen was first activated with crystalline trypsin under conditions leading to the production of primarily δ-chymotrypsin[7]

(5) B. S. Hartley and B. A. Kilby, *Biochem. J.*, **50**, 672 (1952); **56**, 288 (1954); H. Gutfreund, *Discussions Faraday Soc.*, **20**, 167 (1955); H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 656 (1956); *Proc. Natl. Acad. Sci., U. S.*, **42**, 719 (1956); G. H. Dixon, W. J. Dreyer and H. Neurath, *J. Am. Chem. Soc.*, **78**, 4810 (1956); G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1957).

(6) E. F. Jansen, M. D. F. Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949); E. F. Jansen, M. D. F. Nutting and A. K. Balls, *ibid.*, **179**, 201 (1949); E. F. Jansen, M. D. F. Nutting, R. Jang and A. K. Balls, *ibid.*, **185**, 209 (1950); B. R. Stein and K. J. Laidler, *Can. J. Chem.*, **37**, 1272 (1959).

(7) W. J. Dreyer and H. Neurath, *J. Biol. Chem.*, **217**, 527 (1955).

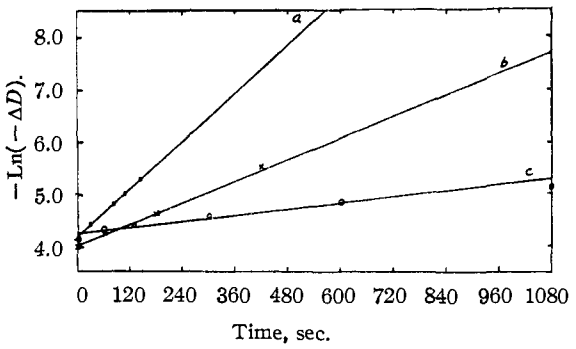

Fig. 2.—First order plot of ΔD_{290} in the reaction of CT with DFP at various (H^+), 0.033 *M* Tris–HCl, 0.033 *M* $CaCl_2$, 17 ± 0.5°. Enzyme concentration in both sample and reference cell, 3.6 × 10^{-5} *M*. At zero time DFP was added to sample cell to give a final concentration of 1.1 × 10^{-4} *M*. Curve a, *p*H 7.5 k' = 75, × 10^{-4} $sec.^{-1}$; curve b, *p*H 9.3, k' = 39 × 10^{-4} $sec.^{-1}$; curve c, *p*H 5.5, k' = 9 × 10^{-4} $sec.^{-1}$.

prior to DFP treatment, a difference spectrum identical in shape and magnitude to that observed in Fig. 1 with CT could be demonstrated.

CT was denatured in 9 *M* urea solution, *p*H 7.0, 25°, for 10 hr. and then treated with a ten fold excess of DFP in 8 *M* urea solution. Here again, a difference spectrum was not observed.

(4) In order to rule out the possibility that the difference spectrum is due to the formation of a reversible enzyme inhibitor complex involving the binding site, analogous samples of DIP-CT and CT were dialyzed for 72 hr. at 1° against several changes of deionized water. Comparison of these dialyzed samples demonstrated the persistance of the spectral peaks (Fig. 5).

(5) Further evidence for the relationship between the specific reaction of DFP or *p*-nitrophenyl acetate with the active site of CT and the difference spectra are the following data.

Figure 2 illustrates first order rate plots of the increase of ΔD_{290} with time at *p*H 5.5, 7.5 and 9.3 at 17°. ΔD in Fig. 2 is the difference between the absorbancy at time t and the absorbancy at the end of the reaction. These data indicate that the rate of the appearance of the difference spectrum is *p*H dependent. The rate of change of ΔD_{290} is greater at *p*H 7.5 than at *p*H 5.5 or at *p*H 9.3. The effect of *p*H on the rate of ΔD_{290} is in agreement with the *p*H dependence of the CT catalyzed hydrolysis of other neutral substrates such as acetyl-L-tryptophanamide.[8] Preliminary experiments indicate that the rate of increase in absorbancy at 290 mμ at *p*H 7.5 (Fig. 2) agrees with the rate of phosphorylation of the enzyme at the same pH and temperature determined by independent means.[9,10]

As demonstrated below, the difference spectrum of monoacetyl-CT *versus* CT disappears as the enzyme becomes deacylated.

(8) D. W. Thomas, R. V. MacAlister and C. Niemann, *J. Am. Chem. Soc.*, **73**, 1548 (1951).

(9) B. J. Jandorf, H. O. Michel, N. K. Schaffer, R. Egan and W. H. Summerson, *Discussions Faraday Soc.*, **20**, 134 (1955).

(10) G. P. Hess and J. M. Sturtevant, unpublished observations, (1960).

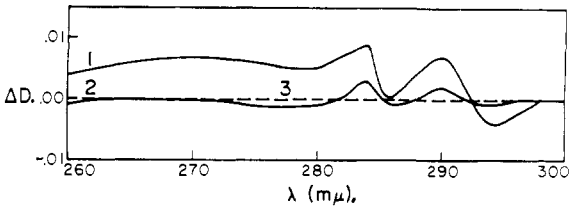

Fig. 3.—Ultraviolet difference spectrum, monoacetyl-α-chymotrypsin *versus* CT; *p*H 6.7 (0.033 *M* Tris–HCl, 0.033 *M* $CaCl_2$), 3.6×10^{-5} *M* enzyme, 25°. Times stated are for absorbancy at 300 mμ. Curve 1, 6 seconds after adjustment of *p*H of monoacetyl-α-chymotrypsin solution from *p*H 3.5 (HCl) to 6.7; curve 2, after 3 minutes; curve 3, after 20 minutes.

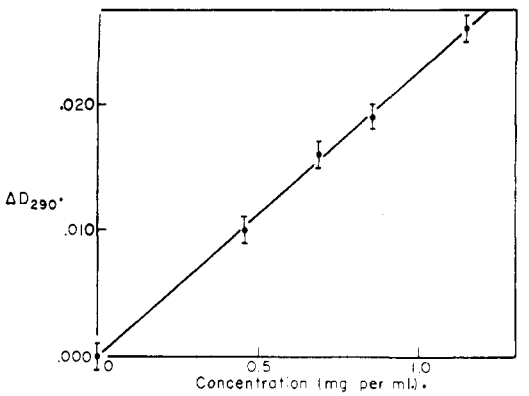

Fig. 4.—Effect of enzyme concentration on ΔD_{290} of difference spectrum of DIP-CT *versus* CT, *p*H 7.5 (0.033 *M* Tris–HCl, 0.033 *M* $CaCl_2$), 25°.

B. **Difference Spectrum of Monoacetyl-CT *versus* CT.**—Monoacetyl-CT, obtained from the reaction of *p*-nitrophenyl acetate with CT at *p*H 5.0 and isolated according to the method of Marini and Hess,[2c] was used in these experiments. The difference spectrum of monoacetyl-α-chymotrypsin *versus* CT, six seconds after raising the *p*H of the monoacetyl-α-chymotrypsin solution from *p*H 3.5 (where the enzyme is stable) to *p*H 6.7, is shown in Fig. 3, curve 1.

The characteristic component of this difference spectrum is a peak at 290 mμ. Although variation in the spectra are observed with alterations in *p*H in the region examined, *p*H 3.0–9.0, the peak at 290 mμ appears, initially, essentially unchanged in position or magnitude. A smaller peak in the 280–285 mμ region varies in size and position with environmental changes.

Above *p*H 5.5 the difference spectrum decreases with time as the enzyme becomes deacylated. This can be seen in Fig. 3, curves 2 and 3. Thus the peak at 290 mμ appears to be directly related to the presence of the acetyl group in the active site of the enzyme.

Characterization of the Difference Spectrum of DIP-CT *versus* CT.—A series of experiments was carried out in order to differentiate between various types of interaction which could result in the spectral shift responsible for the appearance of the difference spectrum.

A. **Effect of Enzyme Concentration on ΔD_{290}.**—If the 290 mμ peak in the difference spectrum is due to a chromophore and not to some form of intermolecular interaction, or to instrumental artifacts, the relationship between ΔD_{290} and enzyme concentration should conform to Beer's Law.[11]

The relationship between ΔD_{290} and enzyme concentration follows Beer's Law up to a concentration of 1.15 mg. per ml. as demonstrated in Fig. 4. The molar extinction difference coefficient calculated from these data is 565 (1 cm. light path), assuming a molecular weight for α-chymotrypsin of 25,000.

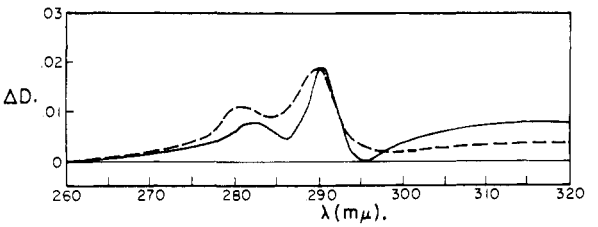

Fig. 5.—Effect of ionic strength on difference spectrum of DIP-CT *versus* CT, 3×10^{-5} *M* enzyme. 25°. Both samples were first dialyzed against deionized water (see text). Solid line, $\mu = 0.5$ *M* in KCl; dashed line, $\mu = 6 \times 10^{-4}$ *M*.

B. **Effect of *p*H and Ionic Strength on Difference Spectrum.**—These experiments were performed to investigate the possibility that the difference spectrum is due to development of a charge near a chromophore in one form of the enzyme and not in the other.

At high ionic strength a charge effect should be dampened, and if it were the cause for the difference spectrum, a decrease in the 290 mμ peak would be expected. The experiment illustrated in Fig. 5 indicates that large changes in ionic strength do not effect either the magnitude or the position of the principal peak at 290 mμ.

Another means of demonstrating that no charge effect is involved is to neutralize positive or negative charges by *p*H adjustment. The difference spectra of DIP-CT *versus* CT were therefore undertaken both in aqueous solution and in 8 *M* urea at various *p*H's from 2 through 12.

Figures 6 and 7 illustrate the difference spectra of DIP-CT *versus* CT obtained at various *p*H values from 2 through 12, both in aqueous solutions and in 8 *M* urea. The 290 mμ spectral peak is clearly demonstrable in all cases, essentially undiminished in intensity. Other spectral effects are superimposed on the characteristic difference spectrum at the extreme *p*H's, particularly in aqueous solution, as shown in Fig. 6. These may be due to differences in the stability of DIP-CT and CT under extreme conditions. A difference in the stability of DIP-CT and CT is clearly demonstrated in the experiments in 8 *M* urea to be discussed below.

The difference spectra at *p*H 9.0 and above were obtained within 1 minute after adding calculated amounts of base to analogous samples of CT and DIP-CT. The actual *p*H of the solutions was measured immediately after the difference spectrum was obtained. These values agreed with the initial *p*H of the solutions obtained in parallel experiments.

(11) I. Fridovich, W. Farkas, G. W. Schwert, Jr., and P. Handler, *Science*, **125**, 1141 (1957).

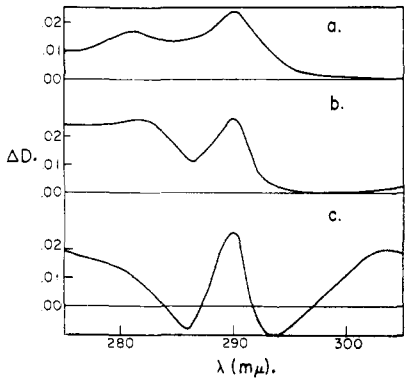

Fig. 6.—Effect of *p*H on difference spectrum of DIP-CT *versus* CT, 3.6×10^{-5} *M* enzyme, $\mu = 0.20$ *M*, 25°. Curve a, *p*H 7.0; curve b, *p*H 2.0; curve c, *p*H 12.0.

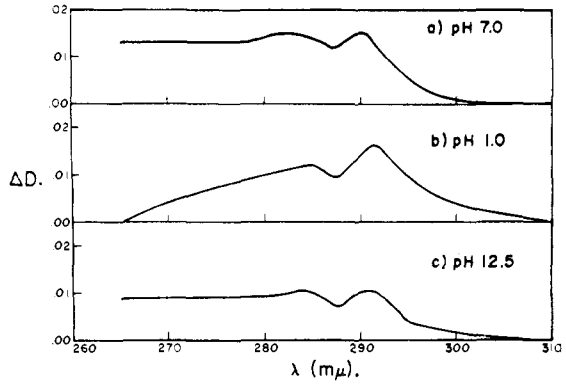

Fig. 7.—Effect of *p*H on difference spectrum of DIP-CT *versus* CT in 8 *M* urea. 3.6×10^{-5} *M* enzyme, 25°. Samples were denatured in 9 *M* urea, *p*H 7.0 at 37° for 48 hr. before adjustment of *p*H. The final urea concentration was 8 *M*. Curve a, *p*H 7.0; curve b, *p*H 1.0; curve c, *p*H 12.5.

Phosphate analysis indicated that the DIP group is stable under the conditions used in these experiments. This is in agreement with the experiments of Whitaker and Jandorf.[12]

C. Effect of Denaturation on Difference Spectrum.—Analogous samples of DIP-CT and CT were made 9 *M* with respect to urea at *p*H 7.0 and 37° and were allowed to stand for various time intervals. The difference spectra were taken in 8 *M* urea solutions. Initially the difference spectrum illustrated in Fig. 8 is obtained. This spectrum has a principal maximum at 292.5 mμ and a smaller peak at 285 mμ. These peaks slowly decrease in magnitude and are no longer discernible after 3 hr. At this time the difference spectrum of DIP-CT *versus* CT in 8 *M* urea solution (Fig. 7, curve a) is similar to the difference spectrum obtained in aqueous solution (Fig. 6, curve a). This difference spectrum is reproducibly observed after analogous samples of DIP-CT and CT were allowed to stand in 9 *M* urea solutions at *p*H 7.0 and 37° for 48 hr. The slight decrease in magnitude observed in 8 *M* urea is consistent with the decrease in magnitude of the absolute spectra of proteins in this solvent.[13]

(12) J. R. Whitaker and B. J. Jandorf, *J. Biol. Chem.*, **223**, 751 (1956).

(13) D. B. Wetlaufer, J. T. Edsall and B. R. Hollingworth, *ibid.*, **233**, 1421 (1958).

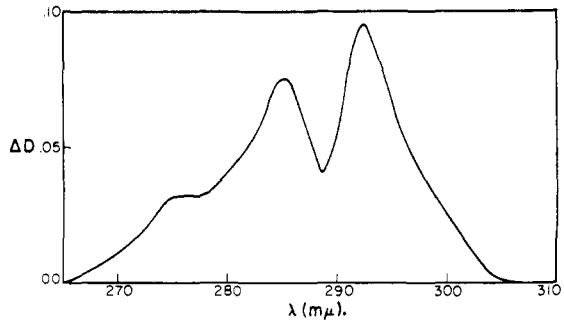

Fig. 8.—Denaturation difference spectrum, DIP-CT *versus* CT, *p*H 7.0, 3.6×10^{-5} *M* enzyme, 25°. Difference spectrum was obtained ten minutes after addition of urea to both solutions to give a final concentration of 8 *M*.

The conditions employed in the experiments illustrated in Fig. 7 should be adequate to insure complete configurational randomization. Using optical rotation changes as a criterion, Neurath, Rupley and Dreyer[14] have demonstrated that both chymotrypsin and chymotrypsinogen are completely denatured in 7 *M* urea at 0° after 2.5 hr. Chervenka[15] has demonstrated that DIP-CT is more rapidly denatured in urea than chymotrypsinogen. Using enzymatic assays as criteria, it has been shown that at *p*H 5.5, 18°, 8 *M* urea inactivates α-chymotrypsin to the extent of 98% in 10 minutes.[2c]

The experiments in Fig. 8 indicate that CT is denatured more rapidly under the above conditions than DIP-CT. This difference spectrum of DIP-CT *versus* CT is obtained at 25° immediately after the addition of urea. According to Chervenka[15] the peaks at 292.5 and 285 mμ are due to denaturation differences. The positive direction of the peaks indicates that the solution in the reference cell, CT, is more rapidly denatured than DIP-CT. These peaks slowly decrease in magnitude and are no longer discernible after 3 hr. when the typical DIP-CT *versus* CT difference spectrum can again be observed (Fig. 7). This difference in stability to denaturing agents appeared again in preliminary experiments on the effect of organic solvents on the DIP-CT *versus* CT difference spectrum. The large peak at 292.5 mμ which appeared in every case in these attempts made observations in the 290 mμ region impossible.

The persistence of the difference spectrum of DIP-CT *versus* CT in 8 *M* urea under the conditions used suggests either the formation of a new chromophore in DIP-CT or a conformation in DIP-CT which resists urea denaturation.

Identification of Origin of Difference Spectrum.—Only two amino acids commonly found in proteins absorb significantly in the 280 to 300 mμ region. CT contains approximately four tyrosyl and seven tryptophyl residues.[16] A shift in the absorption of either of these residues could be responsible for the difference spectrum observed. There-

(14) H. Neurath, J. A. Rupley and W. J. Dreyer, *Arch. Biochem. Biophys.*, **65**, 243 (1956).

(15) C. H. Chervenka, *Biochem. Biophys. Acta*, **31**, 85 (1959).

(16) P. E. Wilcox, E. Cohen and W. Tan, *J. Biol. Chem.*, **228**, 999 (1957).

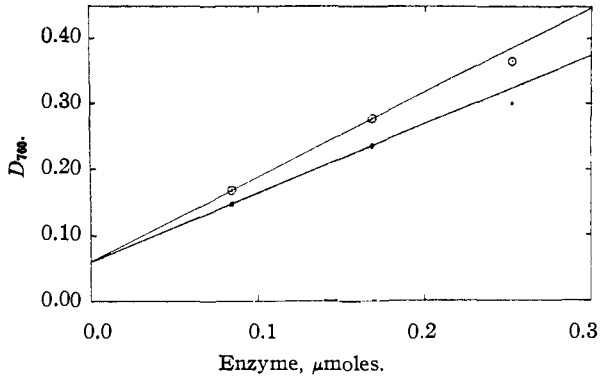

Fig. 9.—Reaction of phenol reagent with DIP-CT and CT at *p*H 8.0. Filled circles, DIP-CT; open circles, CT (see text).

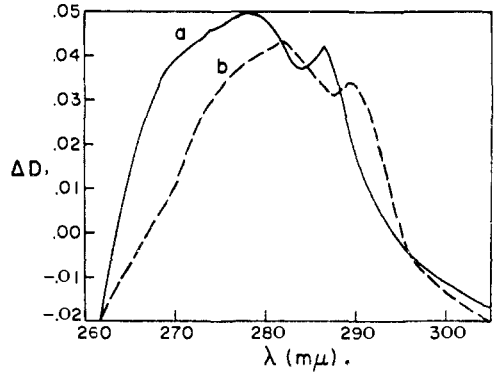

Fig. 10.—Difference spectrum of enzyme samples in 8 *M* urea following treatment with N-bromosuccinimide, 3.6 × 10^{-6} *M* enzyme, 25°. Samples were denatured in 9 *M* urea, *p*H 7.0, at 37° for 12 hr. before reaction. Curve a, difference spectrum of DIP-CT *versus* CT following reaction with identical amounts (≈3.2 × 10^{-4} *M*) of N-bromosuccinimide; curve b, difference spectrum of CT *versus* CT following reaction with different amounts (2.7 × 10^{-4} *M* and 3.2 × 10^{-4} *M*) of N-bromosuccinimide.

fore, it became of interest to determine whether these residues are equivalent in DIP-CT and CT.

A. Reaction with Folin-Ciocalteau Reagent[17] (Phenol Reagent).—It has been demonstrated by Herriot[18] that the phenol reagent will react with tyrosyl and tryptophyl residues in intact proteins at *p*H 8.0. This reagent also reacts with reactive reducing groups such as free sulfhydryls.[19] Chymotrypsin, however, contains no free sulfhydryl groups.[20]

The reaction between phenol reagent and analogous samples of DIP-CT and CT was carried out at *p*H 8.0 and 12.0 in aqueous solutions. The extent of the reaction was measured spectrophotometrically at 760 mμ. The results of the experiment carried out at *p*H 8.0 in aqueous solution are shown in Fig. 9. The common intercept of the two lines on the ordinate corresponds to the average reagent blank of nine experiments run in triplicate. It can be seen that less tyrosine or tryptophan residues react with the phenol reagent in DIP-CT than in CT. In this experiment the molar color equivalent of chymotrypsin solutions at 760 mμ corresponds to only 57 ± 1% of the known amount of tyrosine and tryptophan residues in CT,[16] as calculated from standard curves of tyrosine and tryptophan. Based on the molar color equivalents for free tyrosine and tryptophan, the difference in optical density between CT and DIP-CT was calculated to be equal to 0.99 ± 0.09 mole of tryptophan, or 0.91 ± 0.08 mole of tyrosine, per mole of enzyme in the experiments at *p*H 8.0.

The color value obtained in the reaction of phenol reagent with CT at *p*H 8.0 does not agree with the theoretical amount due to seven tryptophan and four tyrosine residues per mole of enzyme. Masking of the amino acid moieties due to molecular folding probably interferes with this reaction. Nevertheless, the data obtained indicate a striking decrease in reactivity of tyrosine and/or tryptophan residues of DIP-CT with phenol reagent.

After fifteen minutes standing at *p*H 12.0 and subsequent treatment with the phenol reagent, the molar color equivalent of chymotrypsin solutions at 760 mμ corresponded to 95% of the theoretical value. The difference between CT and DIP-CT corresponds to 0.38 ± 0.05 mole of tryptophan or 0.25 ± 0.04 mole of tyrosine per mole of enzyme. The difference in reactivity with phenol reagent between CT and DIP-CT may be smaller at this *p*H than at *p*H 8.0 because of slow hydrolysis of the DIP group.

B. Reaction with N-Bromosuccinimide.—The reaction of N-bromosuccinimide with tryptophan and tryptophyl residues in proteins has been investigated by Witkop, *et al.*[21] These authors followed the reaction of this reagent in aqueous solutions with trypsinogen and trypsin by amino acid analysis and demonstrated that only tryptophyl residues were oxidized under their conditions.[22] In similar experiments with CT, Viswanatha and Lawson[23] noted that in a sample of CT in which five tryptophans were oxidized one tyrosyl residue was also oxidized. The oxidation of tyrosyl residues leads to marked spectroscopic changes at 260 mμ.[24] In the experiments described below, which were carried out in 8 *M* urea solutions, absorbancy changes at 260 mμ were not observed (Fig. 10).

Analogous samples of DIP-CT and CT were reacted with varying amounts of N-bromosuccinimide after the samples were denatured by 9 *M* urea for 12 hr. The reaction with N-bromosuccinimide, using the conditions of Viswanatha, *et al.*,[22] was carried out in 8 *M* urea at *p*H 4.0. The extent of the oxidation of tryptophan residues was measured spectrophotometrically as described by Witkop, *et al.*[21] The enzymes were examined for 1 hr. after addition of N-bromosuccinimide and no further spectral changes could be observed

(17) O. Folin and V. Ciocalteau, *J. Biol. Chem.*, **73**, 627 (1927).

(18) R. M. Herriot, *J. Gen. Physiol.*, **19**, 283 (1935).

(19) H. Fraenkel-Conrat, in "Amino Acids and Proteins," D. M. Greenberg, ed., Charles C. Thomas, Springfield, Ill., 1951, p. 532.

(20) B. S. Hartley, *J. Cell. & Comp. Physiol.*, **54**, Suppl. 1, 203 (1959).

(21) A. Patchornik, W. B. Lawson and B. Witkop, *J. Am. Chem. Soc.*, **80**, 4747, 4748 (1958).

(22) T. Viswanatha, W. B. Lawson and B. Witkop, *Biochem. Biophys. Acta*, **40**, 216 (1960).

(23) T. Viswanatha and W. B. Lawson, *Arch. Biochem. Biophys.*, **93**, 128 (1961).

(24) G. L. Schmir, L. A. Cohen and B. Witkop, *J. Am. Chem. Soc.*, **81**, 2228 (1959); G. L. Schmir and L. A. Cohen, *ibid.*, **83**, 723 (1961).

after 15 minutes. The data in Table I demonstrate that even after 9 *M* urea denaturation the tryptophan residues in DIP-CT reacted to a lesser extent with N-bromosuccinimide than the tryptophan residues in CT. The difference amounts to as much as 0.4 tryptophan residue per mole of enzyme. The data reported in Table I represent some of the coordinates of a curve established by 11 different experiments. In each experiment, run in duplicate, six different amounts of N-bromosuccinimide were used.

TABLE I

OXIDATION OF TRYPTOPHYL RESIDUES OF DIP-CT AND CT BY EQUAL AMOUNTS OF N-BROMOSUCCINIMIDE IN 8 *M* UREA

Moles N-bromosuccinimide used per mole of enzyme	Moles of Tryptophan oxidized per mole of enzyme		
	CT	DIP-CT	Difference
~ 3.0	1.4	1.3	0.1
~ 7.0	3.7	3.4	.3
~ 9.0	4.5	4.1	.4
~10.0	5.1	4.9	.2
~10.0	5.2	5.0	.2
~14.0	6.6	6.6	.0

The reaction of N-bromosuccinimide with analogous samples of DIP-CT and CT was carried out under conditions which are believed to lead to the selective oxidation of tryptophyl residues and to complete denaturation of the enzymes. One would expect, therefore, that all tryptophyl residues are equally reactive. Even in these experiments, recorded in Table I, the tryptophyl residues in DIP-CT are less reactive than those in CT. The maximum difference between CT and DIP-CT appears reproducibly when equal amounts of reagent are added to analogous samples of DIP-CT and CT, and 4.5 tryptophan residues are oxidized in CT but only 4.1 in DIP-CT. These results are reasonable if one tryptophan residue in DIP-CT is oxidized by N-bromosuccinimide at a slower rate than the equivalent residue in CT.

The difference spectrum of DIP-CT *versus* CT in 8 *M* urea, following treatment with identical amounts of N-bromosuccinimide, is shown in Fig. 10, curve a. This reaction led to the oxidation of 4.5 tryptophan residues in CT, but only 4.1 tryptophan residues in DIP-CT. The difference spectrum has the over-all shape of an absolute spectrum of tryptophan with a significant shift in the position and relative magnitudes of the absorption peaks.

In an appropriate control experiment analogous samples of CT in 8 *M* urea were treated with different amounts of N-bromosuccinimide. The difference spectrum between two chymotrypsin samples, in which 4.5 and 4.2 tryptophan residues, respectively, were oxidized, is shown in Fig. 10, curve b. The difference spectrum is almost identical to an absolute spectrum of tryptophan.

The conditions of the experiments and the results recorded in Table I and Fig. 10 strongly suggest that the modified residue in DIP-CT is tryptophan. Suppose we have two compounds, I and II. These compounds are identical except that compound I contains two A chromophores, and compound II contains chromophore A and A′. If we remove one A chromophore from I, then the difference spectrum II versus I should give the absolute spectrum of A′. The difference spectrum of CT with 4.2 tryptophans oxidized *versus* CT with 4.5 tryptophans oxidized (Fig. 10, curve b) remarkably resembles the absolute spectrum of tryptophan, slightly modified by the presence of different amounts of oxidation products. Similarly the spectrum illustrated in Fig. 10, curve a, is modified by the absorption of tryptophan oxidation products in the 280–290 mμ region[21] and cannot be considered an absolute spectrum of the modified tryptophyl residue in DIP-CT. There is also uncertainty about the number of tryptophans modified in DIP-CT. It is also possible that structural differences between DIP-CT and CT exist even after denaturation in 9 *M* urea. Nevertheless, the over-all shape of the spectrum is typical for indole and indole derivatives,[25] and the experiment strongly suggests that the modified chromophore in DIP-CT is tryptophan.

Wood and Balls[26] found that the selective oxidation of one tryptophan residue in chymotrypsin decreased the observed rate of the hydrolysis of substrates by 50%. The enzyme still combines stoichiometrically with specific alkylphosphate inhibitors. The authors concluded that this tryptophyl residue serves an auxiliary function, such as substrate binding, but is not an obligate part of the catalytic site *per se*.

The selective oxidation of one tryptophyl residue of chymotrypsin by N-bromosuccinimide[23,27] gives results identical with those obtained by Wood and Balls.[26] The difference spectrum of DIP-CT (−1 Tryptophan) *versus* CT (−1 Tryptophan) is identical to the difference spectrum of DIP-CT *versus* CT[27] illustrated in Fig. 1. This indicates that the first tryptophan oxidized by N-bromosuccinimide, and presumably the tryptophan oxidized by peroxidase, is not involved in the difference spectrum.

C. Ionization of Tyrosine Residues in DIP-CT and CT.—While the data presented so far strongly suggest that the difference spectrum of DIP-CT *versus* CT is due to the modification of tryptophyl residues in DIP-CT, tyrosyl residues could also contribute to this difference spectrum. Spectrophotometric titration of the tyrosyl groups in CT and DIP-CT[28] strongly suggest that these groups are equivalent in both enzymes.

Discussion

The Origin of the Difference Spectrum.—Combined evidence of four types indicates that the origin of the difference spectrum of DIP-CT *versus* CT is a modification of the properties of an indole nucleus of a tryptophyl residue in DIP-CT. First, the position of the primary peak of the difference spectrum is at 290 mμ. Of all the chromophoric amino acids in α-chymotrypsin, only tyrosine and tryptophan absorb in this region. Secondly, the phenol reaction indicates that either tyrosyl or tryptophyl residues are affected. The third

(25) "Physical Data of Indole and Dihydroindole Alkaloids," 4th Ed., Eli Lilly and Co., Indianapolis 6, Indiana, 1960.

(26) H. N. Wood and A. K. Balls, *J. Biol. Chem.*, **213**, 297 (1955).

(27) H. L. Oppenheimer, Master's Thesis, Cornell University, Ithaca, New York, June 1960.

(28) B. Havsteen and G. P. Hess, *J. Am. Chem. Soc.*, **84**, 448 (1962).

point is the difference in reactivity of tryptophyl residues in DIP-CT with N-bromosuccinimide. With this reagent the difference in amount of tryptophan destroyed between DIP-CT and CT and the presence of a modified tryptophan residue in DIP-CT can be directly demonstrated spectrophotometrically. Finally, spectrophotometric titration experiments indicate that the tyrosyl residues in DIP-CT and CT are equivalent.[28] Therefore, the data available so far indicate that an interaction with one or possibly more tryptophyl residues in DIP-CT, which is not found in CT, gives rise to the difference spectrum.

Possible Interactions with Chromophores Responsible for the Difference Spectrum.—The interaction with one or possibly more tryptophyl residues in DIP-CT, which gives rise to the observed difference spectrum, could be of three general types: (1) charge effects and inductive effects; (2) substitution of the chromophore; (3) non-ionic solvent effects. While at the present there is no definite basis for assigning the difference spectrum to any one or more of the above effects, it is possible to exclude some of the effects.

(1) **Charge and Inductive Effects.**—A charge effect is the most unlikely reason for the appearance of the difference spectrum. The observation that the difference spectrum is not affected by large changes in ionic strength (Fig. 5) or *p*H (Figs. 6 and 7) would appear to eliminate this possibility.

An inductive effect alone as the cause of the difference spectrum also appears unlikely. Presumably the acetyl or DIP group is attached to the β-OH group of a serine residue of α-chymotrypsin and a tryptophan residue is sequentially remote from this group.[29] Experiments with tyrosine, tryptophan and phenylalanine and their derivatives indicate that inductive effects are significant only when the perturbing group is within a few Angstroms of the chromophore.[13,30] Therefore, if an inductive effect operates, the tryptophan residue must be held fairly rigidly in close proximity to the acyl group. This spacial relationship must be retained throughout the *p*H range and even in 9 *M* urea. Since the difference spectrum is stable in 9 *M* urea, an inductive effect alone as the cause of the difference spectrum requires that any configurational changes occur equally in DIP-CT and CT. Therefore, the same segment in DIP-CT and CT must be resistant to urea denaturation. Such a postulated rigid structure in both enzymes is not consistent with the data. CT is considerably less stable than DIP-CT in 8 *M* urea. In addition, the presence of an acyl group alone within a rigid segment of the molecule does not explain the dramatic increase, as compared to CT, in the configurational stability of DIP-CT in urea.

(2) **Substitution of Chromophore.**—The possibility of substitution on the indole chromophore is reasonable in terms of the known spectral properties of indole derivatives. Very minor spectral changes are observed between tryptophan and N-acetyl tryptophan; between 2,3-dimethyl indole and 1-methylol-2,3-dimethyl indole[31]; and tryptamine, 2-methyltryptamine and 2,3-dimethyl indole.[32] However, a direct substitution by the acylating agent is unlikely because the difference spectra of DIP-CT *versus* CT (Fig. 1), monoacetyl-CT *versus* CT (Fig. 3) and cinnamyl-CT *versus* CT[33] (after correction for the absorbancy of the cinnamyl group) appear identical. Therefore, if a substitution of the indole chromophore occurs, it would presumably involve the formation of an internal bond between an amino acid residue and a tryptophyl residue of DIP-CT.

(3) **Non-Ionic Solvent Effects.**—The possibility of the difference spectrum arising due to non-ionic solvent effects is not only reasonable but also presents the simplest explanation of the data. The modified properties of the tryptophyl residue can be merely symptomatic of structural changes which accompany the acylation of CT and result in the formation of hydrophobic bonds with a segment of the molecule which has previously been exposed to solvent. One or more tryptophyl residues would be located in this segment of CT. All of the spectroscopic and chemical data can be explained if a tryptophyl residue is less accessible to solvent in DIP-CT than in CT. The difference spectrum indicates a long wave length shift of the tryptophan spectrum in DIP-CT. The ultraviolet absorption maxima of proteins undergo shifts to longer wave length in going from a denatured protein to a molecule in which the secondary and tertiary structure is intact.[34] This long wave length shift is closely paralleled by changes in the spectra of benzene, indole and phenol, respectively, on passing from water to hydrocarbon solvents.[35] The envelopment of tryptophan residues in a hydrophobic region of the molecule would also explain the decreased chemical reactivity of tryptophan in DIP-CT and the greater stability of DIP-CT toward urea.

If one accepts this interpretation of the data, an interesting conclusion may be reached. Namely, that the tryptophyl residue, which is modified in acylation of CT, is probably located in an amorphous region of the molecule. If this residue were located in a crystalline region of the protein, which is unfolded in urea, one would expect the environment around this residue to change. This would result in a shift of the absolute spectrum of this residue and hence of the difference spectrum. This has not been observed.

The Relationship Between the Difference Spectrum and the Acylation of the Active Site of CT.—The difference spectrum appears only in the reaction of DFP with active CT and not in the reaction with chymotrypsinogen or denatured CT. The

(29) N. K. Schaffer, L. Simet, S. Harsham, R. R. Engle and R. W. Drisko, *J. Biol. Chem.*, **225**, 197 (1957); F. Turba and G. Gundlach, *Biochem. J.*, **327**, 186 (1955); R. A. Oosterbaan, P. Kunst, J. van Rotterdam and J. A. Cohen, *Biochem. Biophys. Acta*, **27**, 549, 556 (1958); J. A. Cohen, R. A. Oosterbaan, H. S. Jansz and F. Berends, *J. Cell. & Comp. Physiol.*, **54**, Suppl. 1, 231 (1959).

(30) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *Biochem. Biophys Acta*, **29**, 455 (1958).

(31) B. G. Edwards, *Arch. Biochem.*, **21**, 103 (1949).

(32) T. Hoshino and K. Tamura, *Ann.*, **500**, 42 (1932).

(33) M. L. Bender, G. R. Schonbaum and C. A. Hamilton, *J. Polymer Science*, **49**, 75 (1961).

(34) G. H. Beaven, E. R. Holiday and E. M. Jope, *Discussions Faraday Soc.*, **9**, 406 (1950); G. H. Beaven and E. R. Holiday, *Adv. Prot. Chem.*, **7**, 319 (1952).

(35) S. Yanari and F. A. Bovey, *J. Biol. Chem.*, **235**, 2818 (1960).

agreement of rates of acylation and deacylation, in the reaction of CT with DFP and *p*-nitrophenyl acetate, with the rates of appearance and disappearance of the characteristic 290 mμ peak is striking. The difference spectrum of acetyl-CT *versus* CT disappears on deacylation of the acetyl-enzyme. The difference spectra have not only been observed as the result of the acylation of the active site by an inhibitor, such as DFP, but also by substrates such as *p*-nitrophenyl acetate or *o*-nitrophenyl cinnamate.[33] Finally, the difference spectrum of DIP-trypsin *versus* trypsin (Fig. 11) is similar to the difference spectrum of DIP-CT *versus* CT.

If structural changes are responsible for the difference spectrum then they may be intimately related to the formation of the enzyme–substrate complex and the catalytic reaction. A change in structure of the active center of an enzyme can constitute an important driving force in substrate hydrolysis and make a major contribution to the large rates observed in enzymatic reactions.

Experimental

Materials. *Enzymes.*—Crystallized, salt free chymotrypsinogen, α-chymotrypsin and trypsin obtained from Worthington Biochemical Corporation, Freehold, N. J., were used without further purification. The preparation of monoacetyl-α-chymotrypsin has been described.[20]

Reagents.—Tris-(hydroxymethyl)-aminomethane (Tris), was obtained from Sigma. Calcium chloride, 2-propanol, potassium chloride, potassium bicarbonate, potassium hydroxide and urea, all analytical reagent grade, were obtained from Mallinckrodt. Anhydrous 2-propanol was prepared by distilling it twice over anhydrous calcium chloride. Diisopropylphosphorofluoridate was a generous gift of Dr. B. J. Jandorf, Biochemical Research Division, Army Chemical Center, Maryland. The diisopropylphosphorofluoridate was diluted to 0.1 *M* with anhydrous 2-propanol. The solution was used at this concentration or diluted with water immediately before use. Phenol Reagent was obtained from the Will Corporation, Rochester 3, New York. Amino Acids were obtained from Mann Research Labs, 136 Liberty Street, New York 6, N. Y.

Methods. Instrument.—A Cary Model 14 self recording spectrophotometer was employed for all spectral and kinetic studies. Temperature control (± 0.5°) was effected by the use of a water jacketed cell holder.

***p*H Determination.**—A Beckman Model G *p*H meter was used for *p*H determinations up to *p*H 9.5. Above this *p*H, a Radiometer *p*H meter, Type TTTI, was used with a Type B glass electrode. *p*H measurements were made with reference to Beckman *p*H 4.0, 7.0 and 9.0 standard buffers.

Difference Spectra.—For each pair of enzyme samples the same pipette was used to add each solution, and extreme care was exercised to prevent concentration discrepancies. Gravimetric trial experiments indicated that total pipetting errors could be kept within limits such that at an optical density of 2.0 at 280 mμ, the two solutions would not differ by more than 0.003 optical density units. Silicone coated pipets were used for addition of buffer. For pipetting of enzyme solutions, acid cleaned transfer pipets were found to be far superior in reproducibility.

Matched samples of DIP-CT and CT were prepared as follows: Solutions of CT, usually about 3 mg. per ml. by weight, were dissolved in water, filtered through Whatman 42 filter paper and adjusted to *p*H 6.9 by addition of 0.1 *M* potassium bicarbonate. Two identical aliquots were pipetted into matched volumetric flasks. To one, DFP in isopropanol was added to afford approximately three times the concentration of chymotrypsin present. To the other, an equivalent amount of isopropanol was added. The DIP-CT sample was allowed to stand at room temperature for 5 minutes before further adjustments described below.

For *pH studies*, predetermined amounts of hydrochloric acid or potassium hydroxide and appropriate amounts of potassium chloride were added to give the desired *p*H and identical ionic strengths, usually 0.20 *M*. The samples were diluted to volume and mixed. The actual *p*H of the samples was determined as described above. At *p*H values above 9.0 the *p*H was determined both before and after the spectrophotometric measurements.

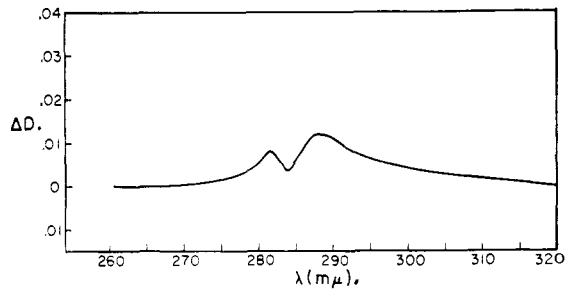

Fig. 11.—Ultraviolet difference spectrum, DIP-trypsin *versus* trypsin, *p*H 7.5 (0.033 *M* Tris-HCl, 0.033 *M* $CaCl_2$), 3.6×10^{-5} *M* enzyme, 3.6×10^{-4} *M* DFP, 25°.

For *studies in urea*, the reaction with DFP was carried out as described above and the matched volumetric flasks were diluted to volume. Four ml. of the CT and DIP-CT solution were pipetted into the 10 ml. volumetric flasks containing 4.805 g. of urea. The flasks were placed in a 37° water bath, swirled to dissolve the urea, and allowed to denature for various times. Then, appropriate additions of acid or base and potassium chloride were made to adjust the *p*H and ionic strength. The samples were diluted to volume and mixed. The actual *p*H of these samples was determined as described above.

Kinetic Studies.—One ml. aliquots of CT-stock solution and 1 ml. of 0.1 M Tris–HCl buffer of appropriate *p*H were added to the sample and reference cell of the Cary Model 14 spectrophotometer. One ml. of distilled water containing isopropanol was added to the reference cell. At zero time 1 ml. of water containing about 3 equivalents of DFP in the same amount of isopropanol as above was added with a syringe type mixing device. Good mixing was obtained and the recording of spectral changes at 290 mμ was begun within 5 seconds. Apparent first order rate constants (k') for the increase in absorbancy were estimated from plots of -ln ($-\Delta D$) *versus* time in seconds. ΔD is the difference between absorbancy at time t and the maximum absorbancy.

Dialysis.—Samples for difference spectroscopy were dialyzed at 1° for 72 hr. in Visking 18/32 seamless cellulose tubing against several changes of deionized water which was agitated by a magnetic stirrer.

Samples for determination of nitrogen and phosphorus content were dialyzed as above against running tap water for 48 hr.

Protein Concentration.—Protein concentrations were determined spectrophotometrically at 280 mμ by using $E^{280}_{1\%} = 20.0$ to relate extinction to protein concentration.[36] The molecular weight of CT was taken as 25,000.[16]

Nitrogen Determination.[37]—Triplicate samples containing 0.5 to 1.5 micro equivalents of nitrogen were digested over night in a sand bath with 1.0 ml. of 2 *N* sulfuric acid containing 0.2 g. of cupric selenite per liter. The samples were cooled and the following reagents were added in order: 2 ml. of water, 2 ml. of Nessler's Reagent and 3 ml. of 2 *N* sodium hydroxide. The samples were allowed to stand for 5 minutes and then read in a Bausch and Lomb Spectronic-20 spectrophotometer at 440 mμ. Ammonium sulfate standard curves were run concurrently for comparison. For calculations of the concentration of chymotrypsin, a molecular weight of 25,000 and a nitrogen content of 16.5% were assumed.[16]

Phosphorus Determinations.[38]—Five ml. samples containing between 12.5 and 100 μg. of phosphorus were run in triplicate. These samples were digested over microburners with 5 ml. of 7.5 *N* sulfuric acid until well charred. After cooling, a few drops of 30% hydrogen peroxide were

(36) G. H. Dixon and H. Neurath, *J. Biol. Chem.*, **225**, 1049 (1957).

(37) M. J. Johnson, *ibid.*, **137**, 575 (1941).

(38) J. B. Sumner, *Science*, **100**, 413 (1944); J. B. Sumner and G. F. Sommers, "Laboratory Experiments in Biological Chemistry," Academic Press, Inc., New York, N. Y., 1949, p. 71.

added, and the samples were boiled until colorless and for an additional 10 minutes to destroy any residual peroxide. The samples were then cooled and rinsed into 50 ml. volumetric flasks. Five ml. of 6.6% ammonium molybdate were added and the samples were diluted to approximately 30 ml. Two ml. of 7.5 *N* sulfuric acid were added, the samples were mixed, and finally, 5 ml. of freshly prepared 8% ferrous sulfate containing 2 ml. of 7.5 *N* sulfuric acid per 100 ml. were added. The samples were diluted to volume, mixed and read at 660 mμ in the Bausch and Lomb spectrophotometer.

Phenol Test.—Samples of CT and DIP-CT containing approximately 2.5, 5.0 and 7.5 mg. of enzyme were run in triplicate.[17,18] One part of phenol reagent was diluted with two parts of water before use.

***p*H 8.0.**—To samples in 25 ml. volumetric flasks were added in order: 6.0 ml. of 0.1 *M* NaCl, 4.0 ml. of 0.4 *M* Na_2HPO_4, 3 ml. of diluted phenol reagent and 2 ml. of 0.9 *M* NaOH. The samples were diluted to volume, mixed and incubated for 15 minutes at 37°. They were then cooled for 5 minutes in a water bath, and the optical density was determined at 760 mμ in a Beckman DU spectrophotometer. The final *p*H of the samples was 8.0. Standard curves were determined using L-tyrosine and L-tryptophan at levels between 0.25 and 1.5 mg. per sample.

***p*H 12.0.**—To the samples in 25 ml. volumetric flasks were added in order: 6.0 ml. of 0.1 *M* NaCl, 3.0 ml. of 0.4 *M* Na_2HPO_4, 3.0 ml. of diluted phenol reagent and 4.0 ml. of 0.9 *M* NaOH. The procedure was continued as at *p*H 8.0.

N-Bromosuccinimide Reaction.[21]—Analogous samples of denatured DIP-CT and CT were prepared as discussed above. After the volumetric flasks were allowed to stand in 9 *M* urea, *p*H 7.0 at 37° for 12 hr. the samples were chilled.

Appropriate solutions of N-bromosuccinimide were prepared in acetonitrile. Immediately before use, 0.5 ml. of the acetonitrile solution was mixed with 4.5 ml. of cold 0.4 *M* acetate buffer, *p*H 4.0. Two ml. aliquots of these N-bromosuccinimide solutions were added to each pair of denatured DIP-CT and CT solution. The samples were then diluted to volume and mixed. The final urea concentration was 8 *M*. The reaction with N-bromosuccinimide was allowed to proceed for at least 15 minutes. Appropriate controls containing acetonitrile-acetate buffer in 8 *M* urea were also prepared.

All samples were scanned in the Cary Model 14 spectrophotometer against appropriate reagent blanks. Difference spectra were scanned between analogous samples of DIP-CT and CT.

The amount of tryptophan destroyed was calculated according to the procedure of Witkop, *et al.*[21]

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

Characterization of the Difference Spectrum of Diisopropylphosphoryl-α-chymotrypsin *versus* α-Chymotrypsin. III. Spectrophotometric Titration of Tyrosyl Groups

BY BENT H. HAVSTEEN[1] AND GEORGE P. HESS

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Two out of four tyrosyl groups of α-chymotrypsin (CT) or diisopropylphosphoryl-CT (DIP-CT) titrate reversibly in 0.70 *M* KCl or in 0.14 *M* KCl. After 3 hr. at *p*H 13 and 16° the remaining two tyrosyl groups can be titrated. The apparent *pK*'s of the reversibly titrating tyrosyl groups of CT and DIP-CT are the same. In a denaturing solvent (5 *M* in guanidine hydrochloride and 1.2 *M* in urea) all four tyrosyl groups in CT and DIP-CT titrate normally with the same *pK*. Correction for the electrostatic interaction of the protein (calculated from the amino acid composition of CT) with the protons of the tyrosyl groups indicates that the *pK*'s of these groups are the same as that of phenol in this solvent. Also, the electrostatic interaction parameter *w*, for the tyrosyl titration in both CT and DIP-CT, indicates absence of any significant *p*H-dependent configurational changes in this solvent in the *p*H region 7–12. Unlike the tryptophyl residues, the tyrosyl residues in CT and DIP-CT appear equivalent.

Investigations of the α-chymotrypsin (CT) catalyzed hydrolysis of *p*-nitrophenyl acetate[2] revealed changes in absorption of the enzyme at 290 mμ,[3] which are intimately related to the formation and decomposition of the acyl-enzyme. These absorption changes have been observed[3] in the catalytic reaction of CT with *p*-nitrophenyl acetate and diisopropylphosphorofluoridate (DFP) and in the reaction of trypsin with DFP. The experiments suggested that the spectral changes are due to reversible conformational changes of the enzyme brought about by acylation of its active site. Presumably these structural changes of the enzyme form an important part of the catalytic process. In order to be able ultimately to identify the part of the CT molecule which participates in these reversible structural changes, initial investigations were concerned with the chemical reactivity of tryptophyl residues in CT and DIP-CT. Both chemical and spectroscopic data indicated[3] that these residues are not equivalent in CT and DIP-CT. This investigation is concerned with the hydrogen ion equilibria of tyrosyl residues in CT and DIP-CT.

Spectrophotometric titration of tyrosyl residues in proteins have been used with excellent results and abnormalities in their behavior due to interactions with other groups have been readily observable.[4] In the experiments reported here the spectrophotometric titrations were carried out in 0.7

(1) Fulbright grantee, 1959–1961.

(2) G. P. Hess and M. A. Marini, *4th Intern. Congr. Biochem. Vienna*, 1958, p. 42; M. A. Marini and G. P. Hess, *Nature*, **184**, 113 (1959); *J. Am. Chem. Soc.*, **81**, 2594 (1959); **82**, 5160 (1960); J. F. Wootton and G. P. Hess, *ibid.*, **82**, 3789 (1960).

(3) G. P. Hess and J. F. Wootton, *Fed. Proc.*, **19**, 340 (1960); J. F. Wootton and G. P. Hess, *Nature*, **188**, 4752 (1960).

(4) M. Laskowski, Jr., J. M. Widom, M. L. McFadden and H. A. Scheraga, *Biochem. Biophys. Acta*, **19**, 581 (1956); H. A. Scheraga, *ibid.*, **23**, 196 (1957); C. C. Bigelow and M. Ottesen, *ibid.*, **32**, 574 (1959); O. O. Blumenfeld and M. Levy, *Arch. Biochem. Biophys.*, **76**, 97 (1958); C. Tanford, J. D. Hauenstein and D. G. Rands, *J. Am. Chem. Soc.*, **77**, 6409 (1955); M. Laskowski, Jr., S. J. Leach and H. A. Scheraga, *ibid.*, **82**, 571 (1960).